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I-Domain-Antigen Conjugate (IDAC) for Delivering Antigenic Peptides to APC: Synthesis, Characterization, and *in vivo* EAE Suppression

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Abstract

The objectives of this work are to characterize the identity of I-domain-antigen conjugate (IDAC) and to evaluate the *in vivo* efficacy of IDAC in suppressing experimental autoimmune encephalomyelitis (EAE) in mouse model. The hypothesis is that the I-domain delivers $PLP_{139-151}$ peptides to antigen-presenting cells (APC) and alters the immune system by simultaneously binding to ICAM-1 and MHC-II, blocking immunological synapse formation. IDAC was synthesized by derivatizing the lysine residues with maleimide groups followed by conjugation with PLP-Cys-OH peptide. Conjugation with PLP peptide does not alter the secondary structure of the protein as determined by CD. IDAC suppresses the progression of EAE while I-domain and GMB-I-domain could only delay the onset of EAE. As a positive control, Ac-PLP-BPI-NH_2-2 can effectively suppress the progress of EAE. The number of conjugation sites and the sites of conjugations in IDAC were determined using tryptic digest followed by LC-MS analysis. In conclusion, conjugation of I-domain with an antigenic peptide (PLP) resulted in an active molecule to suppress EAE *in vivo*.

Introduction

Autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type-1 diabetes are caused by the recognition and attack of self-tissues or organs by the host immune systems. In the case of multiple sclerosis (MS), the immune systems attack the myelin sheath of the neurons causing disruption of the signal translation in the central nervous system (CNS). One of the potential ways that T-cells recognize the myelin sheath is by activation of a subset of autoreactive T-cells, which recognize the self-myelin sheath. One possible mechanism of activation of a subpopulation T-cell is via formation of the "immunological synapse" at the interface between T-cells and antigen-presenting cells (APC). The immunological synapse is a "bull's eye"-like structure that is composed of a cluster of interactions between T-cell receptors (TCR) and major histocompatibility complex-peptide (MHC-p) at the center (Signal-1) and a cluster of interactions between costimulatory molecules (i.e., Signal-2: B7/CD28, ICAM-1/LFA-1) at the periphery of the bull's eye. The differentiation of naïve T-cells to a specific subset (i.e., Th1, Th2, Th17, T-reg) is strongly dependent on the type of co-stimulatory signal being delivered. Blocking Signal-2 during this process could lead to immune unresponsiveness of T cells called

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anergy.[1] Inhibition of ICAM-1/LFA-1 (Signal-2) interaction suppresses Th1-type immune response and could promote a non-inflammatory suppressor and/or regulatory T cells.[2] Potential drugs such as monoclonal antibodies (mAb) or small molecules that block Signal-2 have been developed for treating autoimmune diseases; unfortunately, as a potential side effect, these drugs may suppress the general immune response and compromise the ability of the host to respond to pathogenic infections.

To overcome the general suppression of immune systems, our approach is to modulate the activation of a subpopulation of T-cells that recognizes a specific antigen using a bifunctional peptide inhibitor (BPI).[3] The BPI molecules are composed of an antigenic peptide for the specific disease conjugated to a cell adhesion peptide via a spacer.[4–8] GAD-BPI, PLP-BPI, and CII-BPI molecules have been shown to induce immunotolerance in non-obese diabetes (NOD),[6] experimental allergic encephalomyelitis (EAE),[3, 4, 7, 8] and collagen-induced arthritis (CIA), respectively. The antigenic peptide is derived from epitope of the protein antigen responsible for the autoimmune disease, while the adhesion peptide is derived from either the sequence of LFA-1 (LABL) or ICAM-1 (cIBR1). The potential mechanism of action of BPI molecules is via simultaneous binding to MHC-II and ICAM-1 receptors on the surface of APC, respectively. The simultaneous binding to these two-target receptors prevents the translocation Signal-1 and Signal-2 molecular complexes and inhibits the formation of the immunological synapse. As a result, the BPI molecules suppress the generation of inflammatory T-cells and possibly stimulate the formation of suppressor or regulatory T-cells.

Antigenic spreading has been shown to occur in the late stages of EAE and MS, where the antigenic determinant spreads to different epitopes within an antigenic protein (e.g., proteolipid protein (PLP)) or to epitopes of different antigenic proteins such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Although BPI molecules have excellent efficacy to block the progress of EAE, BPI molecules are made from a single antigenic peptide conjugated to a single cell adhesion peptide. Therefore, BPI molecules may not be able to overcome antigenic spreading in EAE or MS. One way to overcome antigenic spreading is to deliver a mixture of BPI molecules with different antigenic peptides. Another way is to simultaneously deliver several different epitopes of antigenic proteins (e.g., PLP, MBP or MOG) to APC by conjugating them to the I-domain protein to make an I-domain-antigen conjugate (IDAC).

As a proof-of-concept, several $PLP_{139\text{-}151}$ peptides were conjugated via maleimide spacers to several lysine residues on the IDAC molecule (Figure 1 and Table 1). The I-domain is the binding region of LFA-1 to ICAM-1, and it has been shown to interact with the D1 domain of ICAM-1.[9] Compared to the cell adhesion peptide LABL, the I-domain offers a unique divalent cation coordination site called metal ion-dependent adhesion site or MIDAS that interacts with the ICAM-1 D1 domain. Here, PLP-Cys-OH was conjugated to the GMB-I-domain to give IDAC, and the IDAC molecule was characterized by tryptic digest mass spectrometry.[10] The *in vivo* efficacy of IDAC was compared to those of negative control (PBS) and positive control (Ac-PLP-BPI-NH_2-2). The results showed that IDAC can delay the onset of EAE compared to PBS, and that IDAC strongly suppresses the progression of EAE.

Experimental Procedures

Materials

The amino acids used for peptide synthesis were purchased from Peptide International (Louisville, KY). GMBS (*N*-[γ-maleimidobutyryloxy]succinimide ester) was from Pierce

(Rockford, IL). Sequence-grade modified trypsin was from Promega (Madison, WI). All other chemicals or solvents were of analytical grade or better.

Mice

The *in vivo* studies were carried out using female inbred SJL/J (H-2^S) mice purchased from Charles River Laboratories, Inc. (Wilmington, MA). The animals were housed under specific pathogen-free conditions at an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility at The University of Kansas. The protocol for working mice had been approved by the Institutional Animal Care and Use Committee (IACUC).

Peptide synthesis

The sequences of peptides used in the present study are listed in Table 1. The standard Fmoc solid-phase peptide chemistry was used to synthesize all peptides on PEG-PS resin (Applied Biosystems, Foster City, CA) with the automated peptide synthesis system (Pioneer™ perspective Biosystems, Framingham, MA). Peptide synthesis and purification were conducted according to our previously published method.[4] All peptides were purified using semi-preparative C_{18} reversed-phase HPLC, and the purity of each fraction from the preparative HPLC was determined by analytical HPLC. The pure fractions were pooled and lyophilized; the molecular weight of each peptide was confirmed by electrospray ionization mass spectrometry ($M+H^+$) (MW PLP-Cys-OH = 1624.86; Ac-PLP-BPI-NH_2-2 = 3416.95).

Preparation of I-domain

The LFA-1 I-domain protein was over-expressed, refolded, and purified as previously described.[11] The protein purity, identity, and secondary structure were confirmed by SDS-PAGE, mass spectrometry, and far-UV circular dichroism (CD), respectively.

Synthesis of IDAC

As shown in figure 1, two steps are required to prepare the IDAC. The first is to modify the amino groups of the N-terminal and side-chain of lysine residues of I-domain by reacting them with *N*-[γ-maleimidobutyryloxy]succinimide ester (GMBS). This step introduces maleimide groups on the I-domain to generate the GMB-I-domain. The second step is to conjugate the thiol group on the Cys residue of PLP-Cys-OH peptide to the maleimide groups on the I-domain to give IDAC.

Step 1—To a total of 20 mg of I-domain solution, a tenfold molar excess of freshly prepared GMBS (2.71 mg) solution in DMSO (0.5 mL) was added dropwise followed by stirring of the mixture for 1 h at 24 °C. Then, the reaction mixture was subjected to purification through a Superdex 75 column to isolate the GMB-I-domain. The desired GMB-I-domain and the excess GMBS were eluted with PBS containing 10 mM $MgSO_4$. The fractions containing the GMB-I-domain were collected and concentrated by ultrafiltration. Modification on the I-domain using this method produced 3–10 maleimide groups per I-domain as determined by electrospray ionization mass spectrometry (ESI-MS).

Step 2—The conjugation reaction of the PLP-Cys-OH peptide to GMB-I-domain was carried out at pH 8.5 to give IDAC. To a solution containing 10 mg of GMB-I-domain, a 15 molar excess of PLP-Cys-OH dissolved in PBS was added dropwise. During addition of the peptide, the pH was constantly monitored and adjusted to 8.5. During the reaction, the final concentration of the protein was 2.0 mg/mL. The reaction was carried out for 1 h at 24 °C with constant stirring. After the reaction was completed, the solution pH was readjusted to pH 7.4. The resulting IDAC reaction mixture purified using a Superdex 75 column. The

fractions belonging to the IDAC were collected and concentrated by ultrafiltration. IDAC contained 1 to 5 peptide molecules per I-domain molecule as determined by ESI-MS. The purity of the IDAC was confirmed by SDS-PAGE gel and size-exclusion chromatography. The CD spectrum of the IDAC was compared with that of the parent I-domain protein.

LC-ESI-MS analysis of intact protein

To obtain intact protein masses, pure protein samples obtained after SEC were analyzed by LC ESI-Q-TOF MS. HPLC separations were performed with a Water Acquity solvent delivery system using a binary gradient of solvent A composed of 98.92:1:0.08 H_2O/acetonitrile/formic acid (vol/vol/vol) and solvent B containing 98.92:1:0.08 acetonitrile/H_2O/formic acid (vol/vol/vol). Approximately 10 pmol of the sample was loaded onto a C_4 reversed-phase HPLC column (1 × 50 mm, 5 μm, 300 Å; Micro-Tech Scientific, Vista, CA) with a linear gradient from 20% to 60% B in 10 min with a flow rate of 20 μL/min followed by a wash and re-equilibration step. Furthermore, the HPLC system was coupled online to the electrospray source of a Q-TOF-2 mass spectrometer (Micromass UK Ltd., Manchester, U.K.). Mass spectra were acquired with instrument cone voltage 35 eV, collision energy 20 eV with Ar in the collision cell. The instrument was set up in positive reflector mode with a scan time of 5 s and in the mass range *m/z* 700–3000. The instrument was calibrated using NaI. The ion chromatograms were processed to obtain the molecular weights of the modified peptides using MaxEnt1 in the *MassLynx* v 4.1 software (Micromass UK Ltd.).

Gel electrophoresis

The pure protein solution (i.e., 100 μg of IDAC or I-domain) obtained after SEC separation was mixed with a 4X Tris–glycine SDS sample buffer containing no reducing agent and loaded into 1.5-mm-thick 10-well NuPAGE® Novex 4–12% Bis-Tris gradient gels. After running gel electrophoresis at 150 V for 70 min, the gels were stained with 0.25% Coomassie blue R250 solution (10% acetic acid/50% ethanol/40% water) for 30 min followed by destaining (10% acetic acid/25% ethanol/65% water) until the bands were visible and the background was clear.

In-gel trypsin digestion

A standard in-gel protein digestion protocol was followed as described elsewhere.[12] Briefly, protein bands were excised from the gel and were digested with trypsin at an enzyme-to-substrate ratio of 1:25 (w/w) at 37 °C overnight. To stop the digestion, 2 μL of glacial acetic acid was added to each sample.

LC-MS/MS analysis of tryptic-digest products

The products of tryptic digest from I-domain and IDAC were introduced onto a capillary reversed-phase HPLC and CID spectra from peptides were obtained with a hybrid tandem hybrid ion trap/ion cyclotron resonance mass spectrometer (LTQFT ThermoFinnigan, Bremen, Germany) under conditions described previously.[13] The experimental raw data were processed using Bioworks software (Thermo, version 2.0) to create an MS/MS peak list in a DTA format. Protein sequence mapping was performed using Sequest, Mascot (Matrix Science, version 2.2), and X!Tandem (www.thegpm.org) algorithms with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 1.2 Da. Amino groups of lysine residues and protein N-terminus were considered to be modified with maleimide linker moiety + dipeptide (Phe-Cys). The chemical composition of the modification for IDAC is $C_{20}H_{23}N_3O_6S$, delta monoisotopic mass 433.1308 and its maleimide hydrolysis product is $C_{20}H_{25}N_3O_7S$, delta monoisotopic mass 451.1413. Protein modification sites identified by tryptic digestion of the final construct are based on the assumption that trypsin cleaves at the carboxyl side of the lysine residue (^{12}K) of the peptide PLP-Cys-OH leaving a

construct peptide of FC … linker … I domain tryptic peptide (see figure 1 step 2). Scaffold software (Proteome Software Inc., version 2.06) was used to combine and validate MS/MS-based peptide identifications. Peptide identifications with greater than 50% probability as specified by the Peptide Prophet algorithm[14] were accepted for reporting protein coverage.

Induction and suppression of EAE

Disease Stimulation—Female inbred SJL/J mice, 5–7 weeks old, were randomly divided into different groups. All mice were immunized with $PLP_{139–151}$ in Freund's complete adjuvant (CFA) to induce EAE, as reported previously.[3, 4] The PLP/CFA emulsion (50 μL per site) was administered to four separate regions above the shoulder and on the flanks. In addition, 200 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was injected intraperitoneally on day 0 and 2.

In vivo study—After disease stimulation, one group of mice received intravenous (i.v.) injections of 26 nmol/injection/day of the conjugate IDAC on days 4 and 7. The second and third groups of mice received i.v. injections of 100 nmol/injection/day of Ac-PLP-BPI-NH_2-2 and 100 microliter of PBS, respectively, on days 4, 7, and 10. The animals were weighed and observed daily. Disease progression was evaluated observed using a blinded method as reported previously.[3, 4] The clinical scores were rated using the following scale: 0–no clinical signs of disease; 1–tail weakness or limp tail; 2–paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3–paraplegia (complete paralysis of two hind limbs); 4–paraplegia with forelimb weakness or paralysis; and 5–moribund or dead. Mice were euthanized once they were found to be moribund.

Statistical analysis

Statistical differences among the groups in clinical disease scores were determined by calculating the average score for each mouse from the day of disease onset to day 20 by One-way Analysis of Variance followed by Fisher's least significant difference using StatView (SAS Institute, Cary, NC). Statistical differences among the groups in body weight were also analyzed in the same way. The presence of significant difference is denoted with *p*-values of < 0.05 or < 0.001.

Results

Synthesis and characterization of IDAC

IDAC was prepared by conjugating the PLP-Cys-OH peptide to the N-terminus and side chain amino groups of the lysine residues in the I-domain (Figure 1). Thus, the amino groups in the I-domain were reacted with the active N-hydroxysuccinimide (NHS) ester of GMBS to produce GMB-I-domain protein via a stable amide bond (Step 1, Figure 1). The GMB-I-domain from the reaction mixture was purified from the excess GMBS using SEC (Figure 2A). Comparison of CD spectra of GMB-I-domain and I-domain shows that they have similar spectra (Figure 2B), indicating that adding of GMB groups does not alter the secondary structure of the GMB-I-domain.

The composition of pure GMB-I-domain was analyzed by liquid chromatography coupled online with ESI-MS. The charge deconvoluted MS spectrum shows three to nine γ-maleimidobutyryloxy (GMB) groups attached to the I-domain with following masses: 21,178 Da, 21,343 Da, 21,508 Da, 21,674 Da, 21,839 Da, 22,004 Da, and 22,169 Da (top panel, Figure 2C). The differences in mass are 165 Da, which is consistent for a sequential addition of GMB group. The first peak at 21,178 Da corresponds to the I-domain molecular weight conjugated to three GMB groups; therefore, the remaining peaks correspond to the I-domain with four to nine covalently linked GMB groups, respectively. The parent I-domain

has a MW of 20,682 (bottom panel, Figure 2C) and it was not found in the MS spectrum of GMB-I-domain (top panel, Figure 2C).

Along with the desired GMB-I-domain peak, there are corresponding peaks with a mass increase of 18 and 36 Da found in the MS spectra (top panel, Figure 2C). These peaks correspond to the hydrolysis of maleimide groups or maleic acid derivatives, which are attached to the I-domain.[15] The MS data correlate with the SDS-PAGE image of the isolated product of GMB-I-domain from SEC, which shows two bands on lane 4 in Figure 2D. These two bands are from the desired maleimide and maleic acid derivatives of I-domain with different electrophoretic mobility. Before purification, the reaction mixture (lane 3, Figure 2D) shows three bands while the control I-domain (lane 2, Figure 2D) produces only one band. The formation of maleimide hydrolysis products is increased upon storage; thus, it is important that the GMB-I-domain be used within 48 h after SEC purification.

Conjugation of PLP-Cys-OH peptide to GMB-I-domain

PLP-Cys-OH is a peptide that contains $PLP_{139-151}$ sequence with an additional cysteine amino acid at the C-terminus with an open carboxylic acid (Cys-OH). PLP-Cys-OH was reacted with GMB-I-domain at pH 8.5 to prepare IDAC. In this case, the peptide conjugation is via nucleophilic attack of the maleimide groups on the GMB-I-domain by the thiol group of the Cys residue on the peptide (Step 2, Figure 1). Crude products were purified with SEC; the desired IDAC could be easily separated from PLP-Cys-OH (Figure 3A). The pure fractions of IDAC were pooled and concentrated. The crude and purified product was analyzed by SDS-PAGE gel against the parent I-domain (Figure 3B). As expected, the parent I-domain shows one single band with lower molecular weight than the conjugates (lane 2, Figure 3B). The gel of the crude product illustrates the presence of IDAC along with lower MW bands corresponding to PLP-Cys-OH (lanes 3, Figure 3B). The gel of purified IDAC shows multiple bands, which have higher molecular weight than the parent I-domain and without the starting PLP-Cys-OH (lanes 4, Figure 3B). The multiple bands on IDAC were due to various levels of peptide conjugates.

The charge deconvoluted LC-MS data indicated that 0–5 PLP peptides were attached to the IDAC with an average of 2.5 PLP peptides per I-domain (Fig. 3C). Each subpopulation of peaks in the spectra of IDAC has various MW peaks due to the presence of different numbers of GMB groups, but the same number of PLP-Cys peptide attached to the I-domain. The complexity in each of the subpopulations arises from the hydrolysis products of maleimide Figure 4.[15] The CD spectrum of each conjugate was similar to that of the parent I-domain protein (Figure 3D), indicating that conjugation of PLP-Cys peptide to the I-domain preserves the native secondary structure of the protein.

Structural analysis of IDAC

To investigate the structure of IDAC, peptide mapping using tryptic digestion and mass spectrometry was used to determine the location of PLP peptides on the I-domain protein. The assumptions are that the modified lysine residues on the I-domain cannot be cleaved by trypsin, and the cleavage product of the lysine residue that is attached to the PLP peptide can be used to identify the modified lysine residue on the I-domain. The modified peptide fragments were identified using LC-MS/MS. The PLP peptide contains a Lys residue (Lys12), which also could be hydrolyzed by trypsin to produce a dipeptide, Phe-Cys-OH, which is attached to maleimidobutyryloxy. The attached dipeptide has a molecular weight of 433.1307 Da for Phe-Cys-OH. In addition, we observed Phe-Cys-OH conjugated to the peptide fragment via the hydrolysis product of maleimide, which has 18.0106 Da molecular weight added. The comparison of LC-MS/MS data from tryptic-digest fragments of the conjugates is summarized in Table 2. The LC-MS/MS sequence coverage for IDAC was

99% and a total of 15 modified tryptic peptides were identified from IDAC. All of the conjugation sites were partially modified, and the number of unmodified peptides dominated the search profile. These modified peptides were unique and were found in the mapping profiles of the conjugates but not in the parent I-domain profile. The experimental and the calculated mass values of the modified peptides were very close, with the average deviation being less than 0.1 Da.

Suppression of EAE by IDAC

The clinical scores indicate that two injections of IDAC have significant efficacy to suppress the progress of EAE compared to those treated with PBS ($p < 0.0001$, through days 12–17); however, three injections of Ac-PLP-BPI-NH_2-2 provide complete suppression of the disease ($p < 0.0001$, through days 12–17) (Figure 6A). The efficacy of IDAC is also reflected in the ability to prevent the changes of bodyweights of the mice significantly compared to PBS-treated mice (Figure 6B). The disease incidence was delayed in the IDAC-treated mice compared to PBS-treated mice ($p < 0.0001$, through days 12–24) and completely eliminated in Ac-PLP-BPI-NH_2-2-treated mice ($p < 0.0001$, through days 12–24) (Figure 6C).

Discussion

Patients with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and psoriasis are currently being treated with protein-derived drugs such as monoclonal antibodies and peptide polymers, which modulate the immune system. Current treatments for multiple sclerosis patients include Copaxone® and Tysabri® as well as anti-inflammatory agents (*e.g.*, corticosteroids, beta-interferon-1a, mitoxantrone). Copaxone® has side effects in patients, including hypersensitivity reaction, shortness of breath, diarrhea, back and neck pain, and fever. Tysabri,® which binds to the α_4-subunit of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins to block leukocyte adhesion and infiltration into CNS, could cause progressive multifocal leuko-encephalopathy (PML) in patients, a life-threatening complication.[16] Similarly, PML was also observed in patients treated with other cell adhesion inhibitors such as Raptiva® (Efalizumab, CD11a mAb) for psoriasis; thus, this drug was withdrawn from the market.[17, 18] Because Tysabri® and Raptiva® bind to integrins, these antibodies presumably also suppress Signal-2 for T-cell activation in addition to blocking the cell adhesion. Blocking Signal-2 of the immunological synapse formation suppresses the general activation of T-cells that can respond to pathogens such as JC virus that causes PML. Therefore, there is a need to discover a new way to suppress T-cell activation in an antigenic-specific manner without suppressing the general immune responses.

To address the issue of antigen specific disease suppression while preserving the immune system's ability to fight to foreign pathogens, our group developed BPI molecules (GAD-BPI, PLP-BPI, and CII-BPI),[3, 4, 6–8] that were derived from antigenic peptides discovered by others[19–24]. In parallel to BPI molecules, we also developed IDAC molecule by conjugating several antigenic peptides to a single molecule of I-domain. Thus, the advantage of making IDAC is that the I-domain can be used to carry multiple copies of the antigenic peptides to improve the potency of the conjugate. In addition, the I-domain can also be utilized to carry multiple and different antigenic peptides to modulate different subpopulations of antigen-specific T-cells. We have shown that the fluorescence-labeled I-domain binds LFA-1 and is internalized by lymphocytes.[25] Similar to BPI molecules, IDAC conjugates are hypothesized to inhibit the immunological synapse formation during the process of T-cell activation by simultaneous binding of the PLP peptide and I-domain to MHC-II and ICAM-1, respectively, on APC. This simultaneous binding forms a bridge between the two receptors and eventually prevents the translocation and reorganization of Signal-1 and Signal-2.

In the present study, it was found that just two i.v. injections (26 nmol/injection) of IDAC inhibited the onset and progress of EAE more efficiently than PBS. The efficacy of IDAC is less than that of the BPI molecule. Increasing the dose to 50 nmol/injection or more by keeping the frequency of injections the same may further suppress the disease. It is also possible to increase the frequency of administration (3–4 i.v. injections) by keeping the dose constant. The structural analyses of IDAC indicated the molecule contains an average of 2.5 peptide molecules per I-domain molecule. To determine the conjugation sites, both the conjugate and the parent I-domain were subjected to tryptic digestion followed by LC-MS/MS analysis using our previous method.[10] IDAC has a total of 15 lysine residues that are modified by PLP-Cys-OH peptide (Table 2). Our previous studies showed that amidation and acetylation of the respective C- and N-termini of PLP peptide on BPI molecules enhanced the *in vivo* activity of BPI molecules. Recently, it was found that IDAC molecules with PLP peptides conjugated to the I-domain had better *in vivo* efficacy than IDAC with uncapped PLP peptides.

The locations of the modified (red) and unmodified (blue) lysine are indicated in the structure of I-domain (Figure 5). If the activity of IDAC is due to its simultaneous binding to ICAM-1 and MHC-II on the surface of APC, the most active conjugate should accommodate binding to these two receptors. It is known that the I-domain binds to ICAM-1 via its MIDAS region (green, Figure 5). From the model derived from the X-ray structure, it can be predicted that conjugation of PLP peptide at K137 and K142 would be the most probable sites to accommodate simultaneous binding of IDAC to MHC-II and ICAM-1. In the future, the most important site(s) for peptide conjugation will be determined using a single mutation of each Lys residue (i.e., K137 and K142) to a Cys residue followed by conjugation of PLP peptide. In this case, the peptide will be derivatized with a maleimide group. To illustrate, Lys142 can be mutated with Cys142 to give Cys142-I-domain. Then, Cys142-I-domain will be conjugated with PLP peptide to give PLP-Cys142-I-domain, and the *in vivo* activity of this new conjugate will be determined in the EAE mouse model. Using this method, we can pinpoint the conjugation site(s) that is important for the activity of the IDAC molecule. It is possible that multiple sites of peptide conjugation are necessary; in such a case, multiple Cys mutations can be carried out on a single I-domain protein. It has been found that double mutations of the I-domain (i.e., F265S and F292G) would increase the affinity of the mutant 2000-fold to ICAM-1.[26] Thus, IDAC molecules derived from the mutant I-domain could enhance their *in vivo* activity to suppress EAE.

In conclusion, we have shown the proof-of-concept that IDAC has efficacy in inhibiting the progress of EAE in the mouse model. In the future, there is a need to evaluate the mechanism of action of the IDAC molecule by evaluating the *in vivo* cytokine production to determine the balance between inflammatory (Th17 and Th1) T cells and regulatory T cells (T-reg). Furthermore, the side effects may be reduced or eliminated upon optimization of dose, schedule, route, and method of administration. As was recently shown for the BPI molecule, vaccine-like injection can to reduce side effects; therefore, vaccine-like delivery of IDAC will also be explored.[27] To test the possibility of using IDAC molecules to suppress antigenic spreading, the I-domain will be conjugated to different antigenic peptides (PLP, MOG, and MBP) to produce multi-antigen IDAC (MIDAC) and evaluated in EAE mice that are induced by different antigens (i.e., PLP, MOG, or MBP). The hope is that MIDAC molecules may offer a unique approach for the treatment of multiple sclerosis.

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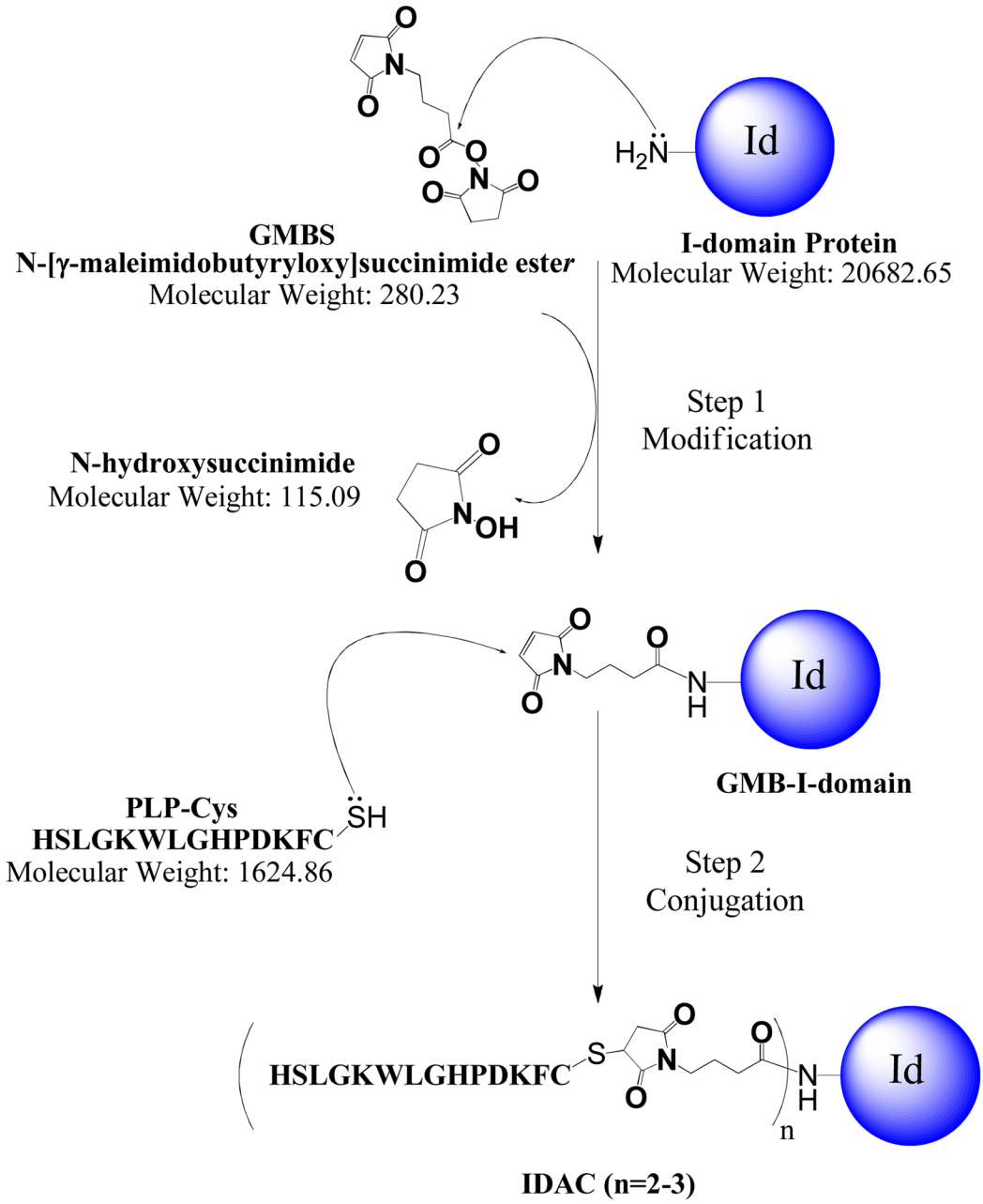

Figure 1.
Schematic representation of a two-step conjugation reaction to prepare IDAC.

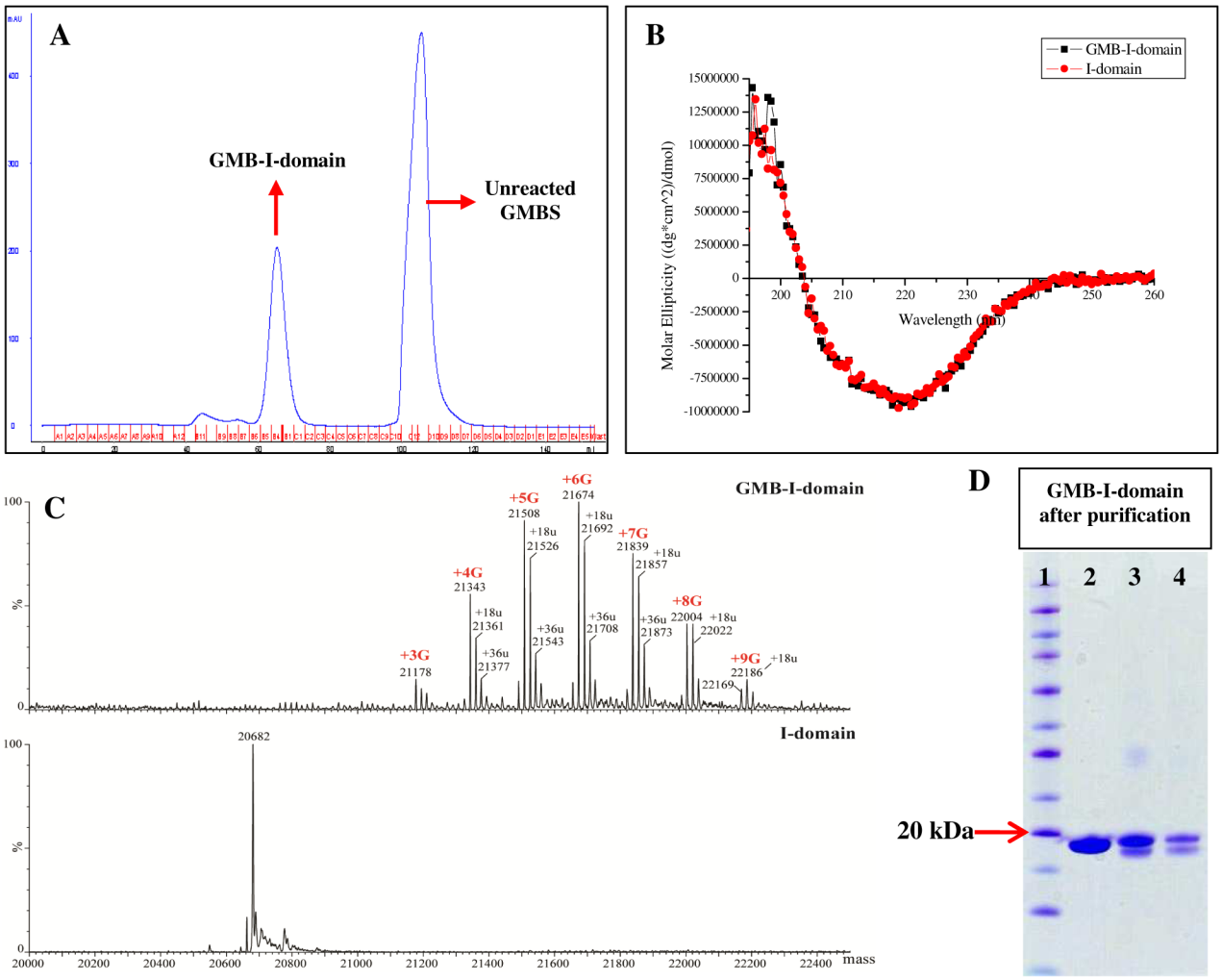

Figure 2.

Purification and characterization of GMB-I-domain after the reaction of I-domain with GMBS. **(A)** SEC chromatogram showing the separation between GMB-I-domain and the remaining free GMBS. **(B)** CD spectra of the parent I-domain (red) and GMB-I-domain (black). **(C)** Deconvoluted mass spectra of LC ESI-MS analysis of the GMB-I-domain protein [8] and the unmodified I-domain protein (bottom). G, is the number of GMBS molecules conjugated. **(D)** SDS-PAGE analysis of pure GMB-I-domain protein after staining with Coomassie blue: molecular weight marker (lane 1), the I-domain protein (lane 2), the reaction mixture of I-domain protein and GMBS (lane 3), and GMB-I-domain protein (lane 4).

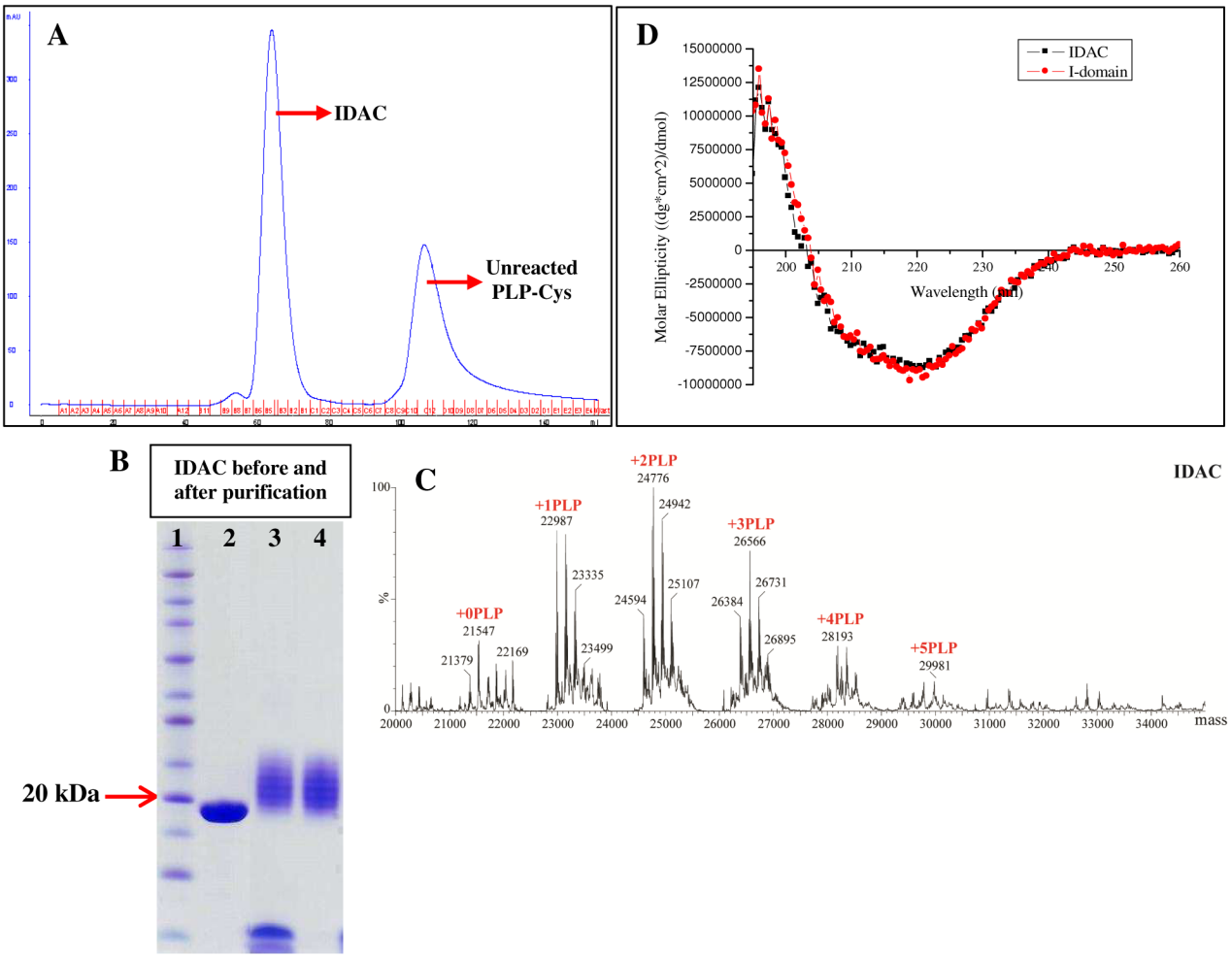

Figure 3.

Purification and characterization of IDAC by SEC, SDS-PAGE, MS, and CD. **(A)** The SEC chromatogram of IDAC, which is separated from the PLP-Cys peptide. **(B)** SDS-PAGE analysis of different proteins after staining with Coomassie blue: molecular weight marker (lane 1), the parent I-domain protein (lane 2), the reaction mixture at pH 8.5 to prepare IDAC (lane 3), and the purified IDAC (lane 4). (C) Charge deconvoluted mass spectra of the IDAC after LC desalting. PLP, is the number of PLP molecules conjugated. **(D)** CD spectra of the parent I-domain (red) and IDAC (black).

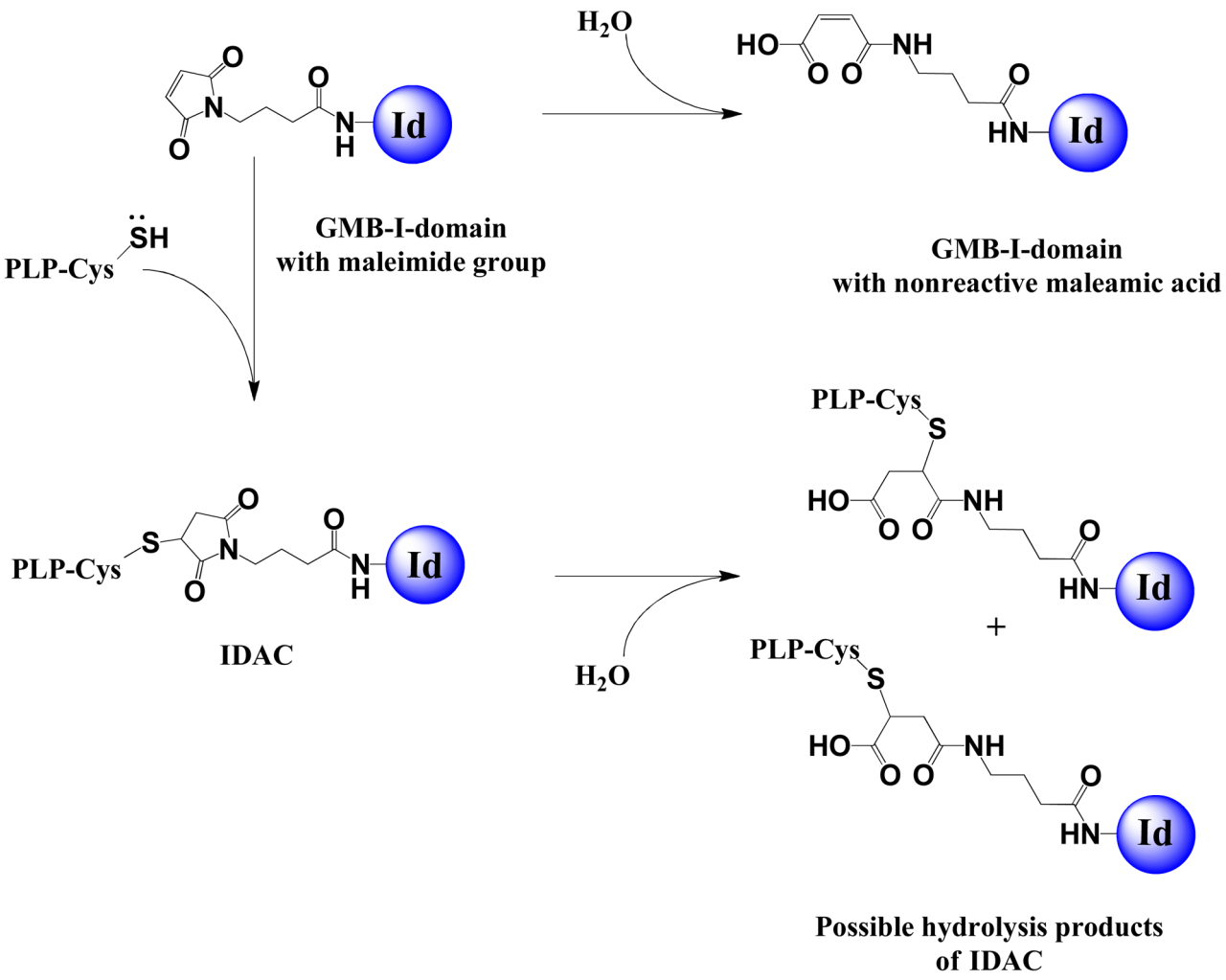

Figure 4.
Schematic representation of possible hydrolysis products of GMB-I-domain and IDAC.

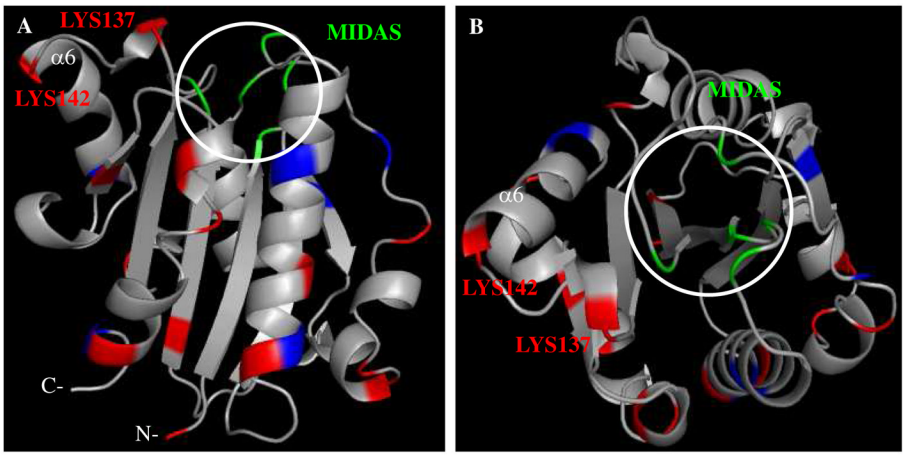

Figure 5.

The X-ray structure of I-domain (PDB code: 1ZON). The residues in red and blue are the respective modified and unmodified lysine sites in IDAC and the residues in green are those located at the MIDAS region. The N- and C-termini are labeled as N- and C-, respectively. The protein images were created using the PyMOL molecular graphics system version 1.4.1 **(A)** Side view. **(B)** Top view.

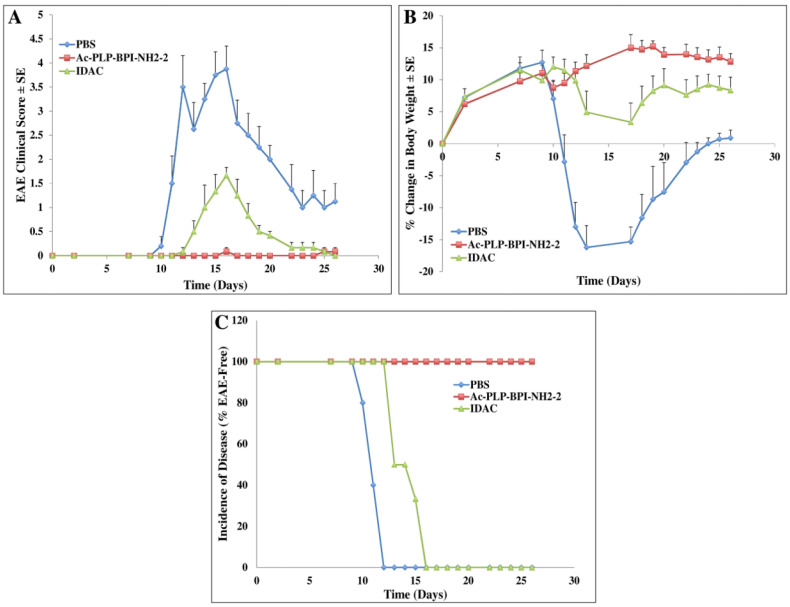

Figure 6.

In vivo activity of IDAC, Ac-PLP-BPI-NH_2-2, and PBS in mouse EAE model. After immunization with PLP peptide in CFA, the mice received i.v. injections of 26 nmol/injection/day of the conjugate IDAC on days 4 and 7. For the Ac-PLP-BPI-NH_2-2 treatment group, the mice received i.v. injections of 100 nmol/injection/day of the peptide on days 4, 7, and 10. The control group was treated with PBS on days 4, 7, and 10. Disease progression was evaluated using **(A)** clinical disease scores, **(B)** change in body weight, and **(C)** incidence of disease. The results are expressed as the mean ± S.E. ($n \geq 6$).

Table 1

List of peptides and proteins used in the present study

Peptide/Protein	Sequence
Ac-PLP-BPI-NH_2-2	*Ac*-HSLGKWLGHPDKF-(AcpGAcpGAcp)$_2$-ITDGEATDSG-*NH_2*
IDAC	(HSLGKWLGHPDKFC)$_n$-linker-I-domain
GMB-I-domain	[*N*-(γ-maleimido)-1-oxybutyl]$_n$-I-domain
I-domain	MGNVDLVFLFDGSMSLQPDEFQKILDFMKDVMKKLSNTSYQFAAVQFSTSYKTEFDFSDYVKRKDPDALLKHVKHMLLLTNTFGAINYVAT

Ac = Acetyl and Acp = Aminocaproic acid

Table 2

Modification sites in IDAC as determined by trypsin digestion and LC-MS/MS

Modified peptide	Sequence	Modified sites
T1	^{1}MGNVDLVFLFDGSMSLQPDEFQ^{23}K	M1
T2-3	^{24}ILDFMKDVM^{33}K	K29
T4-5	^{34}KLSNTSYQFAAVQFSTSY^{52}K	K34
T6-7	^{53}TEFDFSDYVK^{63}R	K62
T8-9	^{64}KDPDALL^{71}K	K64
T9-10	^{65}DPDALLKHV^{74}K	K71
T12-13	^{96}EELGARPDATKVLIIITDGEATDSGNIDAA^{126}K	K106
T13-14	^{107}VLIIITDGEATDSGNIDAAKDII^{130}R	K126
T15-16	^{131}YIIGIGKHFQT^{142}K	K137
T16-17	^{138}HFQTKESQETLH^{150}K	K142
T18	^{151}FASKPASEFV^{161}K	K154
T18-19	^{151}FASKPASEFVKILDTFE^{168}K	K161
T19-20	^{162}ILDTFEKL^{170}K	K168
T20-21	^{169}LKDLFTELQ^{178}K	K170
T21-22	^{171}DLFTELQK^{179}K	K178